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<b>(54) Title:</b> DIAGNOSIS OF $\beta$ -THALASSEMIA USING A MULTIPLEX AMPLIFICATION REFRACTORY MUTATION SYSTEM  <b>(57) Abstract</b>  The present invention provides methods of diagnosing diseases such as $\beta$ -thalassemia using a multiplex amplification refractory mutation system. In addition, kits useful for diagnosing diseases such as $\beta$ -thalassemia are provided.		

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**DIAGNOSIS OF  $\beta$ -THALASSEMIA USING A MULTIPLEX  
AMPLIFICATION REFRACTORY MUTATION SYSTEM**

**CROSS REFERENCE TO RELATED APPLICATIONS**

This application is related to U.S. application  
5 Serial No. 850,703 filed March 13, 1992 entitled "Diagnosis  
of Cystic Fibrosis using Allele Specific Multiplex Polymerase  
Chain Reactions", assigned to the assignee of the present  
application and incorporated by reference in its entirety.  
(Attorney Docket No. CH-0224)

**10 FIELD OF THE INVENTION**

This invention is directed to the diagnosis of  $\beta$ -  
thalassemia using a novel multiplex amplification refractory  
mutation system.

**BACKGROUND OF THE INVENTION**

15 Beta thalassemia is a heterogenous genetic disease  
associated with defective expression of  $\beta$  chain human  
hemoglobin (Hb). Weatherall, et al., *The Thalassemia  
Syndromes* 3rd Ed., (Blackwell, Oxford 1981). Its distribution  
encompasses the Mediterranean basin including North Africa  
20 and the Middle East, as well as China, India and South-East  
Asia. Kazazian, H.H., *Semin Hematol* 27: 209 (1990). More than  
100 mutations have been defined which lead to aberrant  
expression of the  $\beta$ -globin gene in humans.  
Stamatoyannopoulos, G., et al., *The Molecular Basis of Blood  
25 Disease* (W.B. Saunders Co., Philadelphia PA 1987). Of the  
several different types, the IVS-1 nt 1, IVS-1 nt 6, codon  
39, IVS-1 nt 110, IVS-2 nt 1, IVS-2 nt 745 changes are the

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most common mutations among Mediterraneans, and account for approximately 92% of the  $\beta$ -thalassemia defects in this area. Cao, A., et al. *Br. J. Haematol* 71: 309 (1989). A wide variety of strategies and techniques are currently available to detect mutations. Orkin, et al., *Nature* 296: 627 (1982); Saiki, et al., *N. Engl. J. Med* 319: 537 (1988); Losekoot, M., et al., *Br. J. Haematol.* 76 : 269 (1990). However, none appear ideal for rapid screening of large numbers of samples. Foremost issues are detection of as many mutations as possible, cost effectiveness, rapidity and accuracy in obtaining the diagnosis, and eventually, the ability to handle large numbers of samples by automated methods. Methodology should also be simple enough to be utilized in clinical laboratories.

Analyses of DNA frequently take the form of restriction fragment length polymorphism (RFLP) using Southern blotting techniques. While this technique has been useful, it is relatively slow and only allows for the detection of limited number of polymorphic base changes which either create or destroy a restriction endonuclease recognition site.

In addition,  $\beta$ -thalassemia has been diagnosed by using denaturing gradient gel electrophoresis and direct sequencing of PCR amplified genomic DNA. This procedure is limited by the labor intensive nature of the procedure. Thereby increasing cost and decreasing the number of samples which can be tested.

The amplification refractory mutation system is another method which has been used to diagnose  $\beta$ -thalassemia. Varawalla, et al., *Brit. J. of Haematology* 78: 242-247 (1991). This technique involves PCR primers having allele specific 3' nucleotides and corresponding "normal" primers having normal 3' nucleotides. In addition, because in some cases a single 3' allele specific base does not allow amplification to proceed, additional mismatches near the 3' end of appropriate primers are introduced. This procedure was used to screen individually for a number of different  $\beta$ -

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thalassemia mutations including IVS1 nt110, IVS1 nt1, IVS1 nt6, codon 39, codon 6, and IVS2 nt 745. However, simultaneous detection of multiple mutant alleles has not been accomplished. The ability to detect multiple allelic mutations simultaneously is of utmost importance for the detection of as many mutations as possible and for cost effectiveness, rapidity and accuracy in obtaining diagnosis. Furthermore, the detection of multiple mutant alleles is important for the eventual automation of a diagnostic test.

10            Multiplex PCR is another procedure which has been used for the diagnosis of diseases characterized by mutant alleles. Multiplex is useful for the simultaneous amplification of multiple target sequences permitting multiple mutant alleles to be scanned in a single lane of an  
15 agarose gel. This strategy involves appropriate choice of primer pairs so that PCR fragments (either normal or mutant) are generated of different size which can be easily resolved by comparison of samples run in parallel lanes. In males, deletional forms of X-linked diseases such as Lesch-Nyhan  
20 syndrome and Duchenne muscular dystrophy are immediately obvious with this procedure, since missing exons are readily apparent in the amplification pattern. (Gibbs, et al., *Genomics*, 7: 235-244 (1990); Chamberlain, et al., *Nucleic Acid Research* 16: 11141-11156 (1988); Beggs, et al., *Human*  
25 *Genet.* 86: 45-48 (1990). Such a diagnostic tool, effective for detecting multiple allelic mutations, quickly and accurately, is greatly desired for the detection of  $\beta$ -thalassemia.

In particular, a cost-effective, simple, rapid test that  
30 specifically detects the five prevalent mutations of the  $\beta$ -globin gene associated with  $\beta$ -thalassemia would be of major benefit, and could be applied to common mutations in other population groups as well.

#### SUMMARY OF THE INVENTION

35            Methods of diagnosing  $\beta$ -thalassemia which are effective for simultaneously detecting multiple allelic

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mutations quickly and accurately are provided by the present invention. The methods of the present invention comprise the steps of obtaining genomic DNA from a patient suspected of carrying a genetic mutation characteristic of  $\beta$ -thalassemia and selecting at least two primer sets for detecting at least two mutations characteristic of  $\beta$ -thalassemia. Each primer set is comprised of two primer pairs, a first primer pair comprising a specific primer for a normal allele, and a second primer pair comprising a specific primer for a mutant allele. Each pair further comprises a common primer. A polymerase chain reaction is performed in accordance with methods of the present invention using said genomic DNA and said at least two primer sets whereby primer pairs comprising a specific primer for a normal allele are used simultaneously and primer pairs comprising a specific primer for a mutant allele are used simultaneously. Two or more polymerase chain reaction products are detected whereby the detection of a polymerase chain reaction product of a specific primer for a mutant allele indicates the likelihood that said patient carries a mutation characteristic of the phenotype  $\beta$ -thalassemia. In some embodiments of the present invention, each specific primer is differentially labeled. In still other embodiments of the present invention the genomic DNA and all differentially labeled primer sets are used simultaneously to perform the polymerase chain reaction.

In another embodiment of the present invention kits are provided comprising four dNTPs and at least two primer sets selected from the group consisting of

- |    |                   |  |
|----|-------------------|--|
| 30 | IVS-1 nt1-N       | TTAAACCTGTCTTGTAACCTTGATACGAAC (SEQ ID NO: 1), |
|    | IVS-1 nt1-M       | TTAAACCTGTCTTGTAACCTTGATACGAAT (SEQ ID NO: 2), |
|    | and $\beta$ -CRP1 | ACCTCACCTGTGGAGCCAC (SEQ ID NO: 3);            |
|    | IVS-1 nt6-N       | TCTCCTTAAACCTGTCTTGTAACCTTCATA (SEQ ID NO: 4), |
|    | IVS-1 nt6-M       | TCTCCTTAAACCTGTCTTGTAACCTTCATG (SEQ ID NO: 5), |
|    | and $\beta$ -CRP1 | ACCTCACCTGTGGAGCCAC (SEQ ID NO: 3);            |
| 35 | IVS-1 nt110-N     | ACCAGCAGCCTAAGGGTGGGAAAATAGTCC (SEQ ID NO: 6), |
|    | IVS-1 nt110-M     | ACCAGCAGCCTAAGGGTGGGAAAATAGTCT (SEQ ID NO: 7), |
|    | and $\beta$ -CRP1 | ACCTCACCTGTGGAGCCAC (SEQ ID NO: 3);            |
|    | Codon 39-N        | CAGATCCCCAAAGGACTCAAAGAACCTGTG (SEQ ID NO: 8), |

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Codon 39-M CAGATCCCCAAAGGACTCAAAGAACCTGTA (SEQ ID NO: 9),  
and  $\beta$ -CRP1 ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3; and  
IVS-2 nt 1-N AAGAAAACATCAAGGGTCCCATAGACTGAC (SEQ ID NO: 10),  
IVS-2 nt 1-M AAGAAAACATCAAGGGTCCCATAGACTGAT (SEQ ID NO: 11),  
5 and  $\beta$ -CRP1 ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3).

It is therefore an object of the invention to provide methods of diagnosing  $\beta$ -thalassemia. It is a further object of the invention to provide kits useful for diagnosing  $\beta$ -thalassemia. These and other objects will become apparent  
10 by an examination of the detailed description and accompanying claims.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the strategy for multiplex amplification of the areas  
15 encompassing the most common  $\beta$ -thalassemia mutations in Mediterraneans. Approximate locations of the five most common  $\beta$ -thalassemia mutations are indicated ( $\Delta$ ) within the  $\beta$ -globin gene. Multiplex amplification is accomplished using a common upstream primer ( $\beta$ -CRP1) and a mixture selected from  
20 five normal and/or five mutant primers in each polymerase chain reaction. The size of expected PCR products in base pairs is also shown.

Figure 2 shows PCR amplification of normal DNA using normal primers (lanes 2, 4, 6, 8 and 10) or mutant  
25 primers for the same regions (lanes 3, 5, 7, 9 and 11). Multiplex amplification of four regions in normal DNA encompassing IVS-2 nt 1, codon 39, IVS-1 nt 110 and IVS-1 nt 6 with normal primers (lane 12) or mutant primers (lane 13) is also shown. Lanes 14 and 15 show the same pattern of  
30 multiplex allele-specific PCR using the IVS-1 nt 6 normal and mutant primers, respectively. PCR products are sized relative to markers generated from a Hae III digest of  $\phi$ X174 Rf DNA (lane 1). Arrows show location of each PCR product using indicated primers.

35 Figure 3 shows the detection of common Mediterranean  $\beta$ -thalassemia mutations by a multiplex amplification refractory mutation system. All amplifications

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were done with either normal (N) or mutant (M) primer pairs corresponding to the IVS-1 nt 1, IVS-2 nt 1, codon 39, IVS-1 nt 110 and IVS-1 nt 6 regions of the human  $\beta$ -globin gene. Multiplex amplification was done using separate reactions  
5 containing a mixture of either normal or corresponding mutant primer pairs with normal genomic DNA (lanes 10, 11, and 12), DNA from a heterozygote for the IVS-2 nt 1 mutation (lanes 2 and 3), DNA from a heterozygote for the IVS-1 nt 1 mutations (lanes 4 and 5), DNA from a compound heterozygote for the  
10 codon 39 and IVS-1 nt 1 mutations (lanes 6 and 7), DNA from a compound heterozygote for -29 and codon 24 mutations (lanes 8 and 9), respectively. PCR products are sized relative to markers generated from a Hae III digest of  $\phi$ X174 Rf DNA (lane 1).

#### 15 DETAILED DESCRIPTION OF THE INVENTION

Rapid, simple, non-radioactive methods for detection of mutations causing a disease such as  $\beta$ -thalassemia are provided by the present invention. In some methods of the present invention, genomic DNA is obtained  
20 from a patient suspected of carrying a genetic mutation characteristic of a disease such as  $\beta$ -thalassemia. Genomic DNA may be extracted by methods described by Poncz, et al., *Hemoglobin* 6: 27-33 (1982) or with an automated extractor (Applied Biosystems, Inc., Foster City, CA). Other methods  
25 for extraction of genomic DNA known to those skilled in the art are also encompassed by the present invention.

More than 100 mutations have been defined which lead to aberrant expression of  $\beta$ -globin gene in humans. Stamatoyannopoulos, G., et al., *The Molecular Basis of Blood*  
30 *Disease* (W.B. Saunders Co., Philadelphia PA 1987). Some of the most common, accounting for approximately 92% of the  $\beta$ -thalassemia defects in the Mediterranean, are IVS-1 nt 1, IVS-1 nt 6, IVS-1 nt 110, codon 39 and IVS-2 nt 1.

A multiplex amplification refractory mutation  
35 system can be used to detect mutations such as the mutations described above by the appropriate choice of primers. In



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some embodiments of the present invention a primer strategy such as the strategy set forth in Figure 1 can be used to detect at least two  $\beta$ -thalassemia mutations. Primers are designed so that the size of the resulting PCR products  
5 differ, thereby facilitating detection. Oligonucleotide primers of the present invention can be synthesized by procedures known to those skilled in the art such as by solid state phosphoramidite synthesis.

In accordance with methods of the present invention  
10 at least two primer sets for detecting at least two mutations characteristic of a disease such as  $\beta$ -thalassemia are selected. In some embodiments of the present invention four primer sets are selected useful for diagnosing four mutations characteristic of  $\beta$ -thalassemia. In still further preferred  
15 embodiments of the present invention five primer sets are selected which are useful for detecting five mutations characteristic of  $\beta$ -thalassemia. Each primer set is comprised of two primer pairs. A first primer pair is comprised of a  
20 3' primer specific for a normal allele. A second primer pair is comprised of a 3' primer specific for a mutant allele such as an allele specific for  $\beta$ -thalassemia. A mismatched residue is incorporated at the third nucleotide in from the 3' nucleotide, Newton, et al., *Nucleic Acid Research* 17: 2503 (1989), in both normal and mutant 3' primers in order to  
25 ensure selective amplification. The 3' primers differ from each other only at their terminal 3' nucleotide. Thus, for example, the 3' nucleotide of the 3' primer of the first primer pair is substantially complementary to the nucleic acid sequence of a normal allele. The 3' nucleotide of the 3'  
30 primer of the second primer pair may be substantially complementary to the nucleic acid sequence of a  $\beta$ -thalassemia mutation. Each 3' primer has a mismatched incorporated at the third nucleotide in from its 3' nucleotide. Each primer pair further comprises a common primer. Thus, the nucleic  
35 acid sequence of the common primer is the same for both primer pairs comprising a primer set. Under proper annealing temperatures and polymerase chain reaction conditions, these

primers pairs only direct amplification of their complementary allele. For example, normal genomic DNA will be amplified by the first primer pair which is specific for a normal allele. The second primer pair, specific for a mutant allele will not amplify genomic DNA from a normal patient.

In some preferred embodiments of the present invention, each 3' specific primer is differentially labeled, resulting in differentially labeled PCR products. Any label known to those skilled in the art which can be easily differentiated clinically are encompassed by the present invention. For example, dyes known to those skilled in the art may be useful to distinguish PCR products based upon color differentiation. In preferred embodiments of the present invention fluorescent dyes such as FAM™ (blue), JOE™ (green), TAMRA™ (yellow) and ROX™ (red) (Applied Biosystems, Inc., Foster City, CA) may be used. Differential labels may be linked to oligonucleotide primers of the present invention by methods known to those skilled in the art, such as by linker molecules. Linker molecules useful in the present invention may be selected from any of a variety of linker molecules available to those skilled in the art, such as a reactive aminohexyl linker (Aminolink). In other embodiments of the present invention differential label may be incorporated during synthesis of the oligonucleotide primers. In still other embodiments of the present invention, PCR products are labeled by differential recognition by a labeled probe or chemical moiety such as a rhodamine coupled antibody. For example, the specific primer for the IVS-1 nt 1 mutant allele may be labeled with yellow dye and the IVS-1 nt 1 normal allele labeled with blue dye. By detecting a yellow signal, one skilled in the art would be apprised that the patient has a IVS-1 nt 1 mutant allele. A blue signal would indicate a normal allele.

Some primer sets useful in the present invention are set forth in Table I.

TABLE I  
OLIGONUCLEOTIDE PRIMER SETS USED FOR MUTATION DETECTION

Set No.	SEQ. ID NO:	Primer	Coordinates	Primer Type	Sequence	pM
1	1	IVS-1 nt 1-N	+143 to +172	normal	TAAACCTGTCTGTAAACCTTGATACGAAC	90
	2	IVS-1 nt 1-M	+143 to +172	mutant	TAAACCTGTCTGTAAACCTTGATACGAAT	90
	3	$\beta$ -CRP1	-109 to -89	common	ACCTCACCCCTGTGGAGCCAC	90
2	4	IVS-1 nt 6-N	+149 to +178	normal	TCTCCTTAAACCTGTCTGTAAACCTTCATA	10
	5	IVS-1 nt 6-M	+149 to +178	mutant	TCTCCTTAAACCTGTCTGTAAACCTTCATG	10
	3	$\beta$ -CRP1	-109 to -89	common	ACCTCACCCCTGTGGAGCCAC	90
3	6	IVS-1 nt 110-N	+253 to +282	normal	ACCAGCAGCCTAAGGGTGGGAAATAGTCC	20
	7	IVS-1 nt 110-M	+253 to +282	mutant	ACCAGCAGCCTAAGGGTGGGAAATAGTCT	20
	3	$\beta$ -CRP1	-109 to -89	common	ACCTCACCCCTGTGGAGCCAC	90
4	8	Codon 39-N	+298 to +327	normal	CAGATCCCCAAAGGACTCAAAGAACCTGTG	10
	9	Codon 39-M	+298 to +327	mutant	CAGATCCCCAAAGGACTCAAAGAACCTGTA	10
	3	$\beta$ -CRP1	-109 to -89	common	ACCTCACCCCTGTGGAGCCAC	90
5	10	IVS-2 nt 1-N	+496 to +525	normal	AAGAAAAACATCAAGGGTCCCATAGACTGAC	90
	11	IVS-2 nt 1-M	+496 to +525	mutant	AAGAAAAACATCAAGGGTCCCATAGACTGAT	90
	3	$\beta$ -CRP1	-109 to -89	common	ACCTCACCCCTGTGGAGCCAC	90

Coordinates are given relative to the cap site (+1) of the  $\beta$ -globin gene, with "N" indicating normal and "M" mutant primers, respectively.  $\beta$ -CRP1 refers to the common primer having a sequence common to both primer pairs. Bold letters identify 3' mutation and second base change at 3 nucleotides in from the 3' nucleotide. Picomoles (pM) of each primer used per primer pair is also indicated. Sequences are provided in the 5' to 3' direction.

Other primer sets useful to detect a particular disease such as  $\beta$ -thalassemia can be identified using methods known to those skilled in the art. The first member in each set is the specific primer for a normal allele, the second member of each set is the specific primer for the corresponding mutant allele, and the third member of each set is the common primer included in each primer pair.

At least two mutant alleles can be detected simultaneously by methods of the present invention. It is encompassed by some embodiments of the present invention to perform two polymerase chain reactions per diagnosis. In one PCR reaction mixture, primer pairs for normal alleles from each primer set are used. In a second reaction mixture, all primer pairs for mutant alleles from each primer set are used. Thus each polymerase chain reaction in such a diagnostic test causes amplification of genomic DNA using either primers specific for mutant or normal alleles, i.e. half of each primer set per reaction, an entire primer set per diagnosis. Resulting PCR products are run in parallel on gels to detect the presence or absence of bands. For example, diagnosis of  $\beta$ -thalassemia is accomplished in some embodiments of the present invention, by comparison of normal and mutant polymerase chain reaction products. Figure 3, for example, shows four, two PCR diagnoses using five primer sets each. In each diagnosis, one PCR reaction was performed with 3' primers specific for normal alleles (lanes 2,4,6 and 8) and one PCR reaction was performed with primers specific for mutant alleles (lanes 3,5,7 and 9). PCR products run in parallel on a gel confirmed the following genotypes, a

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heterozygote for the IVS-2 nt 1 mutation (comparison of lanes 2 and 3), heterozygote for the IVS-1 nt 1 mutation (comparison of lanes 4 and 5), compound heterozygote for the codon 39 and IVS-1 nt 1 mutations (comparison of lanes 6 and 7). Diagnosis, may be used in the context of the present invention to encompass a procedure whereby two or more primer sets are amplified and interpreted in order to determine the presence or absence of selected normal and mutant alleles in a particular genomic DNA sample. As has been exemplified above, a diagnosis may encompass one or more polymerase chain reactions.

In preferred embodiments of the present invention both members of each primer pair of at least two primer sets are used simultaneously in a single polymerase chain reaction which is run on a single lane of a gel. Differential labels, as described above, are useful herein for distinguishing polymerase chain reaction products, especially those having similar mobilities. Thus PCR products can be distinguished by mobility and label. In addition, labels, such as fluorescent labels may be particularly amenable to automated methods.

In some preferred embodiments of the present invention at least two of the primer sets provided in Table I are selected. It is still more preferred in some embodiments of the present invention to select all of the primer sets of Table I. In some embodiments of the present invention the primer sets 1 (IVS-1 nt 1 N and M), 3 (IVS-1 nt 110 N and M), 4 (Codon 39 N and M) and 5 (IVS-2 nt 1 N and M) are selected. In still other embodiments of the present invention the primer sets 1 (IVS-1 nt 1 N and M), 3 (IVS-1 nt 110 N and M) and 4 (Codon 39 N and M) in accordance with some embodiments of the present invention. In still other embodiments of the present invention the primer sets 3 (IVS-1 nt 110 N and M) and 4 (Codon 39 N and M) are selected.

Kits are also provided by the present invention comprising four dNTPs and at least two primer sets selected from the primer sets provided in Table I.

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The following examples are illustrative, but should not be construed as limiting the present invention.

**EXAMPLES****EXAMPLE 1****5 DNA Samples**

DNA samples were obtained from normal controls and patients either homozygous or heterozygous for the common  $\beta$ -thalassemia Mediterranean mutations. Genomic DNA was extracted using protocols previously described in Poncz,  
10 M.D., et al., *Hemoglobin* 6: 27-33 (1982) or with an automated extractor (ABI, Foster City, CA). Genotypes were confirmed either by DNA sequence analysis using PCR based, cycle-sequencing approach employing laser-activated fluorescence-emission DNA sequencer; Tamary, et al., *Amer. J. Hemat.* in  
15 press (1992); Trifillis, et al., *Blood* 78: 3298 (1991); McBride, et al., *Clin Chem* 35: 2196 (1989); or by denaturing gradient gel electrophoresis. Diazani, et al., *Genomics* (in press), Losekoot, et al., *Br. J. Haematol* 76: 269 (1990).

**EXAMPLE 2****20 Primer Synthesis**

Unlabelled oligonucleotide primers were prepared on a 380B DNA Synthesizer (ABI, Foster City, CA) by the phosphoramidite method at 0.2 mmol scale with (2-O-cyanoethyl)-phosphoramidites; Caruthers, M.H., et al., *Methods in*  
25 *Enzymol.* 154: 287-313 (1987); and were then purified. Oligonucleotide primers were prepared for fluorescent labeling following standard phosphoramidite chemistry preparation by attachment of a reactive aminohexyl linker group (Aminolink) to the 5' end of the primer. Draper, D. and  
30 L.E. Gold, *Biochemistry* 19: 1774-1781 (1980). Following cleavage from the solid support, and deprotection, each modified primer was then reacted with an N-hydroxyl succinimide ester derivative of a distinct dye. ABI 370 user bulletin (1989). The dye labeled primer was then removed  
35 from excess reactants via high performance liquid

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chromatography purification. Sequences of the oligonucleotide primers are as provided in Table I.

### EXAMPLE 3

#### Polymerase Chain Reaction

5 PCR was performed according to methods previously described by Saiki, R.K., et al., *Science* 239: 487-489 (1988). Reaction mixtures (25 $\mu$ l) contained 100ng of genomic DNA, 1.5  $\mu$ M of each dNTP, the common primer and normal and/or mutant primers in a buffer containing 6.7mM MgCl<sub>2</sub>, 16.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5.0  $\mu$ M  $\beta$ ME, 6.8 mM EDTA, 67.0 mM Tris HCl pH 8.8, 10% (v/v) DMSO. The mixture was heated at 95°C for 5 minutes to denature the DNA, and then quickly chilled on ice. Taq DNA polymerase (1.5 U, Perkin Elmer, Norwalk, CT) was added before overlaying the samples with 25  $\mu$ l of mineral oil. The 15 samples were then subjected to 30 cycles on a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) with denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. The last cycle had a 5 minute extension at 72°C. Approximately 15 $\mu$ l of the PCR 20 product were then analyzed following electrophoresis on a 3% (w/v) agarose (NuSieve GTG) gel.

### EXAMPLE 4

#### Selectivity of Oligonucleotide Primers

Differently sized PCR products were produced based 25 upon the strategy illustrated in Figure 1. Unlabelled normal and mutant PCR primers were synthesized so their terminal 3' nucleotide corresponds to either a normal or mutant sequence. An additional mismatched residue was also incorporated at 3 nucleotides in from the 3' nucleotide, Newton, et al., 30 *Nucleic Acid Research* 17: 2503 (1989), in both normal and mutant specific primers in order to ensure selective amplifications. Primers were tested with normal and available mutant DNA samples under stringent PCR conditions to ensure that selective amplification occurred with either normal or 35 mutant primers. The strategy for multiplex generation of

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differently sized PCR products was accomplished using a single common primer located at -109 base pairs upstream of the  $\beta$ -globin gene. Sequence of this primer was identical to the normal sequence. Poncz, et al., *J. Biol. Chem.* 258: 11599 (1982). Normal and mutant products were scored by the presence or absence of correctly sized bands following electrophoresis on agarose gel. PCR was performed in accordance with Example 3.

Separate amplifications using normal primers for each of the five regions in normal DNA gives single bands of expected size (Figure 2, lanes 2,4,6,8 and 10). No amplification products were seen with the mutant primer set (Figure 2, lanes 3,5,7,9 and 11). Multiplex amplification of 4 regions in normal DNA encompassing IVS-2 nt 1, codon 39, IVS-1 nt 110 and IVS-1 nt 6 with normal primers (lane 12) or mutant primers (lane 13) yields the expected combined pattern. Lanes 14 and 15 show the same pattern of multiplex allele-specific PCR using the IVS-1 nt 6 normal and mutant primers respectively.

## EXAMPLE 5

### Diagnosis of $\beta$ -Thalassemia

Different known  $\beta$ -Thalassemia genotypes were used to test the accuracy of the method. Unlabeled mutant and normal PCR products IVS-1 nt 1 and IVS-1 nt 6 are too similar in size to be resolved using this multiplex method. Thus, in order to test the primers four separate PCR reactions were performed in accordance with Example 3. Four mutations were tested by doing two separate PCR reactions. Each reaction contained the common primer and either four mutant or four normal primers for the IVS-1 nt 1, IVS-1 nt 110, codon 39 and IVS-2 nt 1 mutations. Screening for the IVS-1 nt 6 mutations was done in two separate PCR reactions (with normal and mutant primer). Data are shown in Figure 3 confirming the following genotypes, a heterozygote for the IVS-2 nt 1 mutation (comparison of lanes 2 and 3), heterozygote for the IVS-1 nt 1 mutation (comparison of lanes 4 and 5), compound



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heterozygote for the codon 39 and IVS-1 nt 1 mutations (comparison of lanes 6 and 7).

#### EXAMPLE 7

#### Diagnosis of Five Common $\beta$ -Thalassemia Mutations using

#### 5 Fluorescently Labeled Primers

Diagnosis is carried out as provided in Example 5, except that both normal and mutant allele primers are combined in a single PCR reaction mixture. Each reaction contains the common primer and five mutant and five normal  
10 primers for the IVS-1 nt 1, IVS-2 nt 1, IVS-1 nt 6, codon 39 and IVS-1 nt 110 regions of the human  $\beta$ -globin gene. Primers are labeled as follows: IVS-1 nt 1-N (blue), IVS-1 nt 1-M (green), IVS-1 nt 6-N (yellow), IVS-1 nt 6-M (red), IVS-1 nt 110-N (blue), IVS-1 nt 110-M (green) Codon 39-N (yellow),  
15 Codon 39-M (red), IVS-2 nt 1-N (blue), IVS-2 nt 1-M (green). A fluorescently labeled marker lane is run in a separate lane to facilitate sizing PCR products. The PCR product is then analyzed following electrophoresis on a 3% (w/v) agarose (NuSieve GTG) gel using a multi-line argon ion laser such as  
20 a GENE SCANNER™ (Applied Biosystems, Inc. Foster City, CA) to detect the fluorescently labeled PCR products. Multicolored bands indicate heterozygosity for an allele, while single colored bands indicate homozygosity for an allele.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Fortina, Paolo  
Surrey, Saul
- (ii) TITLE OF INVENTION: DIAGNOSIS OF B-THALASSEMIA USING A  
MULTIPLEX AMPLIFICATION REFRACTORY MUTATION SYSTEM
- (iii) NUMBER OF SEQUENCES: 11
- (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: Woodcock Washburn Kurtz Mackiewicz and Norris  
(B) STREET: One Liberty Place - 46th Floor  
(C) CITY: Philadelphia  
(D) STATE: PA  
(E) COUNTRY: U.S.A.  
(F) ZIP: 19103
- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: Johnson, Philip S.  
(B) REGISTRATION NUMBER: 27,200  
(C) REFERENCE/DOCKET NUMBER: CH-0225

(ix) TELECOMMUNICATION INFORMATION:  
 (A) TELEPHONE: 215-569-3100  
 (B) TELEFAX: 215-568-3439

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 30 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTAAACCTGT CTTGTAACT TGATACGAAC

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 30 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTAAACCTGT CTTGTAACT TGATACGAAT

- 18 -

20

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACCTCACCCCT GTGGAGCCAC

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCTCCCTTAA CCTGCTCTGT AACCTTCATA

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid

30

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30

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCTCCTTAAA CCTGTCTTGT AACCTTCATG

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ACCAGCAGCC TAAGGGTGGG AAAATAGTCC

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- 20 -

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACCAGCAGCC TAAGGTGGG AAAATAGTCT

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CAGATCCCCA AAGGACTCAA AGAACCTGTG

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CAGATCCCCA AAGGACTCAA AGAACCTGTA

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AAGAAAACAT CAAGGTCCC ATAGACTGAC

30

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AAGAAAACAT CAAGGTCCC ATAGACTGAT

30

What is claimed is:

1. A method of diagnosing  $\beta$ -thalassemia comprising the steps of:
  - obtaining genomic DNA from a patient suspected of carrying a genetic mutation characteristic of the phenotype  $\beta$ -thalassemia;
  - selecting at least two primer sets for detecting at least two mutations characteristic of  $\beta$ -thalassemia, each set being comprised of two primer pairs, a first primer pair comprising a specific primer for a normal allele, and a second primer pair comprising a specific primer for a mutant allele, each pair further comprising a common primer;
  - detecting two or more polymerase chain reaction products whereby the detection of a polymerase chain reaction product of a specific primer for a mutant allele indicates the likelihood that said patient carries a mutation characteristic of the phenotype  $\beta$ -thalassemia.
2. The method of claim 1 wherein at least four primer sets are selected.
3. The method of claim 1 wherein at least five primer sets are selected.
4. The method of claim 1 wherein the step of selecting at least two primer sets further comprises selecting primer sets in which each specific primer is differentially labeled.
5. The method of claim 4 wherein the step of performing a polymerase chain reaction further comprises using said genomic DNA and all differentially labeled primer sets simultaneously.
6. The method of claim 1 wherein the step of selecting further comprises selecting primer sets comprising



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a first, second and third member, the first member being a specific primer for a normal allele, the second member being a specific primer for a mutant allele, and the third member being a common allele from the group consisting of:

- 5 IVS-1 nt1-N TTAAACCTGTCTTGTAACCTTGATACGAAC (SEQ ID NO: 1),  
 IVS-1 nt1-M TTAAACCTGTCTTGTAACCTTGATACGAAT (SEQ ID NO: 2),  
 and  $\beta$ -CRP1 ACCTCACCCCTGTGGAGCCAC (SEQ ID NO: 3);
- 10 IVS-1 nt6-N TCTCCTTAAACCTGTCTTGTAACCTTCATA (SEQ ID NO: 4),  
 IVS-1 nt6-M TCTCCTTAAACCTGTCTTGTAACCTTCATG (SEQ ID NO: 5),  
 and  $\beta$ -CRP1 ACCTCACCCCTGTGGAGCCAC (SEQ ID NO: 3);
- IVS-1 nt110-N ACCAGCAGCCTAAGGGTGGGAAAATAGTCC (SEQ ID NO: 6),  
 IVS-1 nt110-M ACCAGCAGCCTAAGGGTGGGAAAATAGTCT (SEQ ID NO: 7),  
 and  $\beta$ -CRP1 ACCTCACCCCTGTGGAGCCAC (SEQ ID NO: 3);
- 15 Codon 39-N CAGATCCCCAAAGGACTCAAAGAACCTGTG (SEQ ID NO: 8),  
 Codon 39-M CAGATCCCCAAAGGACTCAAAGAACCTGTA (SEQ ID NO: 9),  
 and  $\beta$ -CRP1 ACCTCACCCCTGTGGAGCCAC (SEQ ID NO: 3); and
- IVS-2 nt 1-N AAGAAAACATCAAGGGTCCCATAGACTGAC (SEQ ID NO: 10),  
 IVS-2 nt 1-M AAGAAAACATCAAGGGTCCCATAGACTGAT (SEQ ID NO: 11),  
 and  $\beta$ -CRP1 ACCTCACCCCTGTGGAGCCAC (SEQ ID NO: 3).

- 20 7. The method of claim 1 wherein the polymerase chain reaction is performed by using said genomic DNA and the primer sets:

- 25 IVS-1 nt1-N TTAAACCTGTCTTGTAACCTTGATACGAAC (SEQ ID NO: 1),  
 IVS-1 nt1-M TTAAACCTGTCTTGTAACCTTGATACGAAT (SEQ ID NO: 2),  
 and  $\beta$ -CRP1 ACCTCACCCCTGTGGAGCCAC (SEQ ID NO: 3);
- IVS-1 nt6-N TCTCCTTAAACCTGTCTTGTAACCTTCATA (SEQ ID NO: 4),  
 IVS-1 nt6-M TCTCCTTAAACCTGTCTTGTAACCTTCATG (SEQ ID NO: 5),  
 and  $\beta$ -CRP1 ACCTCACCCCTGTGGAGCCAC (SEQ ID NO: 3);
- 30 IVS-1 nt110-N ACCAGCAGCCTAAGGGTGGGAAAATAGTCC (SEQ ID NO: 6),  
 IVS-1 nt110-M ACCAGCAGCCTAAGGGTGGGAAAATAGTCT (SEQ ID NO: 7),  
 and  $\beta$ -CRP1 ACCTCACCCCTGTGGAGCCAC (SEQ ID NO: 3);
- Codon 39-N CAGATCCCCAAAGGACTCAAAGAACCTGTG (SEQ ID NO: 8),  
 Codon 39-M CAGATCCCCAAAGGACTCAAAGAACCTGTA (SEQ ID NO: 9),  
 and  $\beta$ -CRP1 ACCTCACCCCTGTGGAGCCAC (SEQ ID NO: 3); and
- 35 IVS-2 nt 1-N AAGAAAACATCAAGGGTCCCATAGACTGAC (SEQ ID NO: 10),  
 IVS-2 nt 1-M AAGAAAACATCAAGGGTCCCATAGACTGAT (SEQ ID NO: 11),  
 and  $\beta$ -CRP1 ACCTCACCCCTGTGGAGCCAC (SEQ ID NO: 3).

- each of said primer sets being comprised of a first, second and third member, the first member being a specific primer  
 40 for a normal allele, the second member being a specific

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primer for a mutant allele, and the third member being a common allele.

8. The method of claim 7 wherein the polymerase chain reaction is performed by using said genomic DNA and said primer sets, the said first and second members of each primer set further being differentially labelled.

9. The method of claim 8 wherein the polymerase chain reaction is performed by using said genomic DNA and all differentially labeled primer sets simultaneously.

10. The method of claim 1 wherein the polymerase chain reaction is performed by using said genomic DNA and the primer sets:

IVS-1 nt1-N TTAACCTGTCTTGTAACCTTGATACGAAC (SEQ ID NO: 1),  
IVS-1 nt1-M TTAACCTGTCTTGTAACCTTGATACGAAT (SEQ ID NO: 2),  
15 and  $\beta$ -CRP1 ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3);  
  
IVS-1 nt110-N ACCAGCAGCCTAAGGGTGGGAAAATAGTCC (SEQ ID NO: 6),  
IVS-1 nt110-M ACCAGCAGCCTAAGGGTGGGAAAATAGTCT (SEQ ID NO: 7),  
and  $\beta$ -CRP1 ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3);  
  
Codon 39-N CAGATCCCCAAAGGACTCAAAGAACCTGTG (SEQ ID NO: 8),  
Codon 39-M CAGATCCCCAAAGGACTCAAAGAACCTGTA (SEQ ID NO: 9),  
20 and  $\beta$ -CRP1 ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3); and  
  
IVS-2 nt 1-N AAGAAAACATCAAGGGTCCCATAGACTGAC (SEQ ID NO: 10),  
IVS-2 nt 1-M AAGAAAACATCAAGGGTCCCATAGACTGAT (SEQ ID NO: 11),  
25 and  $\beta$ -CRP1 ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3).

each of said primer sets being comprised of a first, second and third member, the first member being a specific primer for a normal allele, the second member being a specific primer for a mutant allele, and the third member being a common allele.

11. The method of claim 10 wherein the polymerase chain reaction is performed by using said genomic DNA and said primer sets, the said first and second members of each primer set further being differentially labelled.

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12. The method of claim 11 wherein the polymerase chain reaction is performed by using said genomic DNA and all differentially labeled primer sets simultaneously.

13. The method of claim 1 wherein the polymerase chain reaction is performed by using said genomic DNA and the primer sets:

IVS-1 nt1-N      TTAAACCTGTCTTGTAACCTTGATACGAAC (SEQ ID NO: 1),  
IVS-1 nt1-M      TTAAACCTGTCTTGTAACCTTGATACGAAT (SEQ ID NO: 2),  
and  $\beta$ -CRP1      ACCTCACCTGTGGAGCCAC (SEQ ID NO: 3);

10    IVS-1 nt110-N    ACCAGCAGCCTAAGGGTGGGAAAATAGTCC (SEQ ID NO: 6),  
IVS-1 nt110-M    ACCAGCAGCCTAAGGGTGGGAAAATAGTCT (SEQ ID NO: 7),  
and  $\beta$ -CRP1      ACCTCACCTGTGGAGCCAC (SEQ ID NO: 3); and

15    Codon 39-N      CAGATCCCCAAAGGACTCAAAGAACCTGTG (SEQ ID NO: 8),  
Codon 39-M      CAGATCCCCAAAGGACTCAAAGAACCTGTA (SEQ ID NO: 9),  
and  $\beta$ -CRP1      ACCTCACCTGTGGAGCCAC (SEQ ID NO: 3).

each of said primer sets being comprised of a first, second and third member, the first member being a specific primer for a normal allele, the second member being a specific primer for a mutant allele, and the third member being a common allele.

14. The method of claim 13 wherein the polymerase chain reaction is performed by using said genomic DNA and said primer sets, the said first and second members of each primer set further being differentially labelled.

15. The method of claim 14 wherein the polymerase chain reaction is performed by using said genomic DNA and all differentially labeled primer sets simultaneously.

16. The method of claim 1 wherein the polymerase chain reaction is performed by using said genomic DNA and the primer sets:

IVS-1 nt110-N    ACCAGCAGCCTAAGGGTGGGAAAATAGTCC (SEQ ID NO: 6),  
IVS-1 nt110-M    ACCAGCAGCCTAAGGGTGGGAAAATAGTCT (SEQ ID NO: 7),  
and  $\beta$ -CRP1      ACCTCACCTGTGGAGCCAC (SEQ ID NO: 3); and

35    Codon 39-N      CAGATCCCCAAAGGACTCAAAGAACCTGTG (SEQ ID NO: 8),

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Codon 39-M CAGATCCCCAAAGGACTCAAAGAACCTGTA (SEQ ID NO: 9),  
and  $\beta$ -CRP1 ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3).

each of said primer sets being comprised of a first, second  
and third member, the first member being a specific primer  
5 for a normal allele, the second member being a specific  
primer for a mutant allele, and the third member being a  
common allele.

17. The method of claim 16 wherein the polymerase  
chain reaction is performed by using said genomic DNA and  
10 said primer sets, the said first and second members of each  
primer set further being differentially labelled.

18. The method of claim 17 wherein the polymerase  
chain reaction is performed by using said genomic DNA and all  
differentially labeled primer sets simultaneously.

15 19. A kit comprising four dNTPs and at least two  
primer sets selected from the group consisting of:

IVS-1 nt1-N	TTAAACCTGTCTTGTAACCTTGATACGAAC (SEQ ID NO: 1),
IVS-1 nt1-M	TTAAACCTGTCTTGTAACCTTGATACGAAT (SEQ ID NO: 2),
and $\beta$ -CRP1	ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3);
20 IVS-1 nt6-N	TCTCCTTAAACCTGTCTTGTAACCTTCATA (SEQ ID NO: 4),
IVS-1 nt6-M	TCTCCTTAAACCTGTCTTGTAACCTTCATG (SEQ ID NO: 5),
and $\beta$ -CRP1	ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3);
IVS-1 nt110-N	ACCAGCAGCCTAAGGGTGGGAAAATAGTCC (SEQ ID NO: 6),
IVS-1 nt110-M	ACCAGCAGCCTAAGGGTGGGAAAATAGTCT (SEQ ID NO: 7),
25 and $\beta$ -CRP1	ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3);
Codon 39-N	CAGATCCCCAAAGGACTCAAAGAACCTGTG (SEQ ID NO: 8),
Codon 39-M	CAGATCCCCAAAGGACTCAAAGAACCTGTA (SEQ ID NO: 9),
and $\beta$ -CRP1	ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3); and
30 IVS-2 nt 1-N	AAGAAAACATCAAGGGTCCCATAGACTGAC (SEQ ID NO: 10),
IVS-2 nt 1-M	AAGAAAACATCAAGGGTCCCATAGACTGAT (SEQ ID NO: 11),
and $\beta$ -CRP1	ACCTCACCCTGTGGAGCCAC (SEQ ID NO: 3).

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20. A method of diagnosing a disease characterized by multiple allelic mutations comprising the steps of:

obtaining genomic DNA from a patient suspected of carrying a genetic mutation characteristic of the disease;

5 selecting at least two primer sets for detecting at least two mutations characteristic of the disease, each set being comprised of a first primer pair comprising a specific primer for a normal allele, a second primer pair comprising a specific primer for a mutant allele, each specific primer  
10 being differentially labeled, each pair further comprising a common primer;

performing a polymerase chain reaction by using said genomic DNA and said at least two primer sets whereby primer pairs comprising a specific primer for a normal allele  
15 are used simultaneously and primer pairs comprising a specific primer for a mutant allele are used simultaneously; and

detecting one or more polymerase chain reaction products wherein detection of a polymerase chain reaction  
20 product of a specific primer for a mutant allele indicates the likelihood that said patient carries a mutation characteristic of the disease.

21. The method of claim 20 wherein at least 4 primer sets are selected.

25 22. The method of claim 20 wherein at least 5 primer sets are selected.

23. The method of claim 20 wherein the step of selecting at least two primer sets further comprises selecting primer sets in which each specific primer is  
30 differentially labelled.

24. The method of claim 23 wherein the step of performing a polymerase chain reaction further comprises using said primer sets simultaneously.

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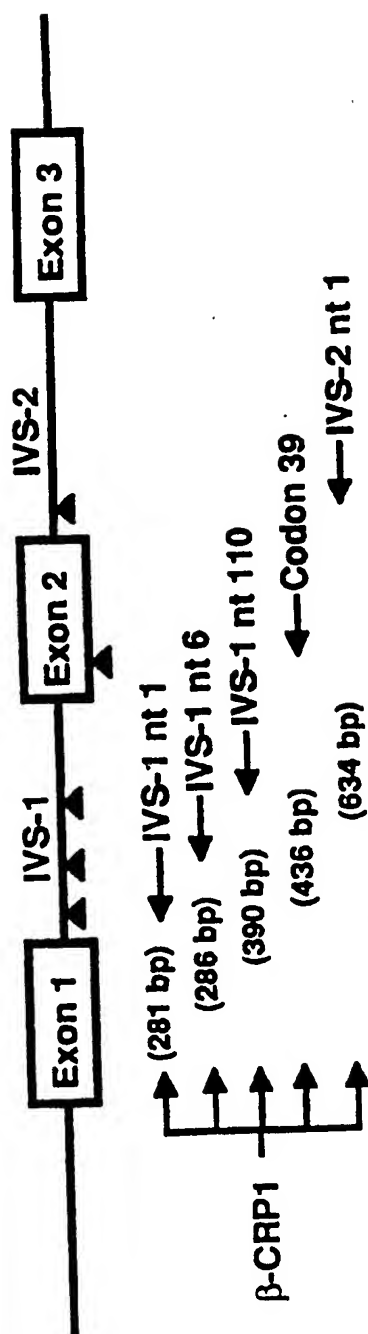


FIG.1

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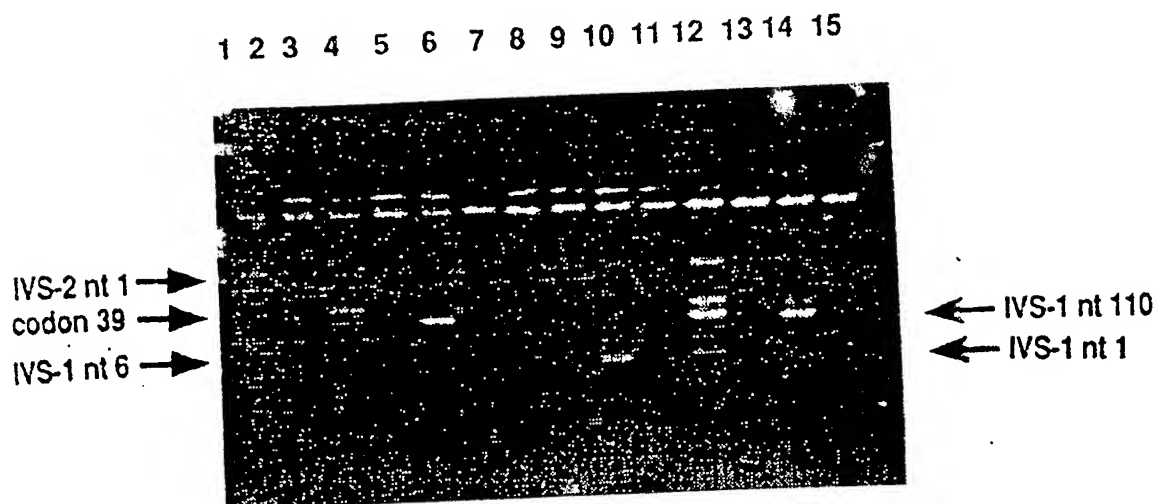


FIG.2

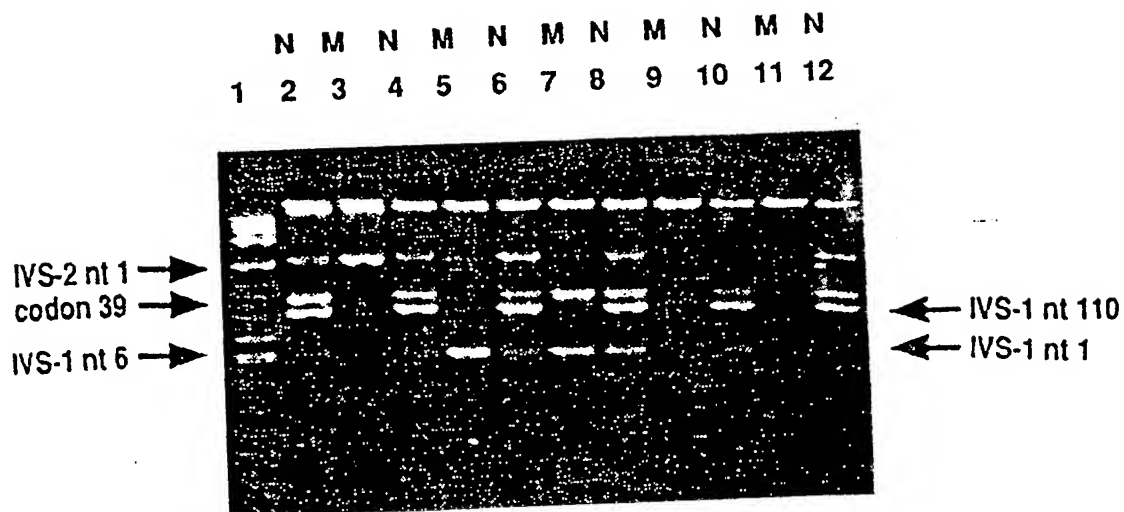


FIG.3

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/02260

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12P 19/34; C07H 21/04

US CL : 435/91; 536/24.33

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91; 536/24.3, 24.33; 935/76, 77, 78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CA, BIOSIS, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proc. Natl. Acad. Sci., Volume 86, issued April 1989, (U.S.A), D.Y. Wu et al., "Allele-Specific Enzymatic Amplification of Beta-globin Genomic DNA for Diagnosis of Sickle Cell Anemia", pages 2757-2760, see page 2758 and Figure 1.	1-18, 20-24
Y	Genomics, Volume 7, issued 1990, R.A. Gibbs et al., "Multiplex DNA Deletion Detection and Exon Sequencing of the Hypoxanthine Phosphoribosyltransferase Gene in Lesch-Nyhan Families", pages 235-244, see page 236.	1-18, 20-24

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

22 April 1993

Date of mailing of the international search report

13 MAY 1993

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/02260

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	British Journal of Haematology, Volume 78, issued 1991, N.Y. Varawalla et al., "The Spectrum of Beta-thalassaemia Mutations on the Indian Subcontinent: the Basis for Prenatal Diagnosis", pages 242-247, see page 243 and Table 1.	1-24
Y	Genomics, Volume 2, issued 1988, M.H. Skolnick et al., "Simultaneous Analysis of Multiple Polymorphic Loci Using Amplified Sequence Polymorphisms (ASPs)", pages 273-279, see entire document.	1-18, 20-24